

Biological approach to reduce *Listeria*

Bacteriophages to control *L. monocytogenes* on ready-to-eat meat products

Listeria monocytogenes is known to be a significant threat to the safety of ready-to-eat (RTE) meat products. Effective hygiene measures can reduce both the frequency and level of contamination of ready-to-eat products with *Listeria monocytogenes*. However, despite these measures, consumers worldwide are often exposed to low numbers of *L. monocytogenes*. Formulating meat products with antilisterial chemicals is a common approach to control *L. monocytogenes* in RTE meat products. An alternative biological and safe approach to reduce the risk for listeriosis makes use of bacteriophages and has recently been introduced by EBI Food Safety under the name Listex™ P100. The effectiveness of this method to eliminate *Listeria*-contamination on pre-packaged sliced cooked meat products was proven through research carried out at Ghent University. The study did not only prove the effectiveness but revealed that a minimal P100-dose of 10^7 pfu/cm² is required to meet the legislative criteria for *Listeria monocytogenes*.

By Lieve Vermeiren,
F. Devlieghere, Dirk de Meester,
Marc Schellekens, Steven Hagens
and Johan Debevere

Bacteriophages (phages) are bacterial viruses that use only specific bacterial cells as their host for replication. Phages are submicroscopic particles that typically consist of nucleic acid (DNA or RNA) surrounded by a protein coat (HUDSON et al., 2005). They are abundant in the environment, in the human gastro-intestinal tract, in water and in food products, inclusive meat and meat products (ATTERBURY et al., 2003).

Bacteriophages have been classified as either lytic phages (virulent and always employ the lytic pathway), or lysogenic phages (temperate). Both types are only able to replicate after infecting a bacterial cell (obligate parasites). In the case of the lytic pathway, infection results in imminent death of the bacterial cell by lysis to release new phage particles. In the case of lysogeny, infection does not result in cell death or cell lysis but results in the integration of the phage genome into the bacterial chromosome. Being part of the bacterial chromosome, the phage genome is replicated during bacterial growth, however no phage particles are formed.

Some phages are strictly lytic while others can switch between the lytic cycle and a lysogenic status and vice versa, following certain stimuli (THIEL, 2004; HUDSON et al., 2005).

For use on food products, lytic phages are preferred. In this lytic pathway (1) phages attach to cell surface receptor molecules of a specific host bacterium, (2) the phage genome is injected into the cell, (3) the bacterial genome is disrupted and the bacterium is killed, as some of the bacteriophage genes are encoding for enzymes that turn off or even destroy the host's DNA, (4) the viral genome is replicated and genes are transcribed and further translated to form the proteins of the phages' capsid, (5), assembly of new phages, up to several hundred per cell, and (6) their endolysins degrade the peptidoglycan of the bacterial cell wall, which results in the release of the phages and death of the host by lysis. In this process, the bacterial cell is destroyed. This cycle may last only 30 to 60 minutes (THIEL, 2004).

Bacteriophages are attractive candidates for protecting food products against proliferation of food pathogens because they are self-perpetuating organisms designed to kill living bacterial cells. They have an inherent host speci-

ficity, that is based on the specific binding between elements of their capsid and specific molecules on the surface of their target bacteria and this ensures that they do not infect eukaryotic cells and even non-target bacterial cells. This means that pathogens can be removed and beneficial organisms (starter cultures, gastro-intestinal flora of the consumer or the background flora of the treated food product) stay. Another valuable quality is that they do not change the organoleptic properties of the foodstuffs.

Listeria monocytogenes is a widely distributed opportunistic foodborne pathogen causing listeriosis. This is a rare disease but of great concern due to its high human case fatality risk (BELL and KYRIAKIDES, 2005). Major concerns with *L. monocytogenes* are its high mortality rate, wide distribution on raw products, growth at low temperatures and its ability to establish itself in various food processing environments. *L. monocytogenes* is well-adapted to survival on equipment and in production facilities and its occurrence in cooked meat products (CMP) is mainly related to post-heat-processing contamination during removal from the cooking containers, slicing, packaging or garnishing. UYTENDAELE et al. (1999) found that incidence rates for CMP were higher after slicing (6.65%) than before slicing (1.65%).

The incidence of *L. monocytogenes* on anaerobically packaged sliced CMP has been reported by several authors. In a survey of CMP on the Belgian retail market (UYTENDAELE et al., 1999), the overall incidence of *L. monocyto-*

genes in 25 g was 4.90% (167/3405). However, only a small proportion (0.53%) of samples contained high contamination levels (>10 cfu/g). The incidence rate was higher for minced CMP (e.g. pâté) than for whole CMP (e.g. cooked ham, cooked poultry), 6.14% and 3.96%, respectively. According to DE BOER (1990), luncheon meat, ham and cooked chicken breast are the most frequently contaminated CMP in The Netherlands. DE BOER and VAN NETTEN (1990) found 11% (9/83) of pâté samples to be contaminated with *L. monocytogenes*. RIJPPENS et al. (1997) reported an incidence of *L. monocytogenes* of 2.6% in 3065 samples of pâté, which was in good agreement with the contamination level of 2.76% (217 samples) in the survey of UYTENDAELE et al. (1999). A more recent study on the occurrence of *Listeria* on sliced cooked meat products in Spain reported an incidence of *L. monocytogenes* in 25 g of 8.8% (35/396) (VITAS and GARCIA-JALON, 2004).

Proliferation of the pathogen on CMP has been demonstrated, although the extent of its growth depends on several factors including temperature, pH, water activity, level of lactate, composition of the headspace atmosphere and the presence of a competitive flora on a product. Growth of *L. monocytogenes* in CMP has resulted in outbreaks of listeriosis. Belgian pâté was the vehicle of infection in listeriosis in the UK, between 1987 and 1989 with more than 350 cases and more than 90 deaths. In 2000, an outbreak of listeriosis in New Zealand following the consumption of cooked

| | | |
|--|--|------------------------------------|
|  | <p>IFFA</p> <p>We look forward to your visit !</p> <p>Hall 8.0, Booth B92</p> | <p>THE POWER OF TECHNOLOGY</p> |
|--|--|------------------------------------|

meats (ham and corned beef) and in the US following the consumption of delicatessen turkey meat was responsible for 60 cases in total. In 2001, precooked sliced turkey was implicated in 10 cases of listeriosis (US) (BELL and KYRIAKIDES, 2005).

Public and regulatory concern related to *L. monocytogenes* has led to the implementation of microbiological standards, aiming at regulating the levels of *L. monocytogenes* in food products (BELL and KYRIAKIDES, 2005). Since 1st of January 2006, an EU regulation on microbiological criteria for foodstuffs has come into force (Commission Regulation (EC) No 2073/2005) (European Commission, 2005). This regulation sets a maximum level of 100 cfu/g for *L. monocytogenes* at the end of the shelf-life of ready-to-eat foods (including CMP). Driven by this new regulation, the search for innovative strategies to control *L. monocytogenes* has received even more interest than before. In view of the consumer trends towards natural and healthy food products, the use of bacteriophages as a means of inactivating foodborne pathogens (HUDSON et al., 2005) has been proposed. DYKES and MOORHEAD (2002) have reported phage control of *L. monocytogenes* in meat. They investigated the effect of listeriophage LH7 on the growth and survival of two strains of *L. monocytogenes* on

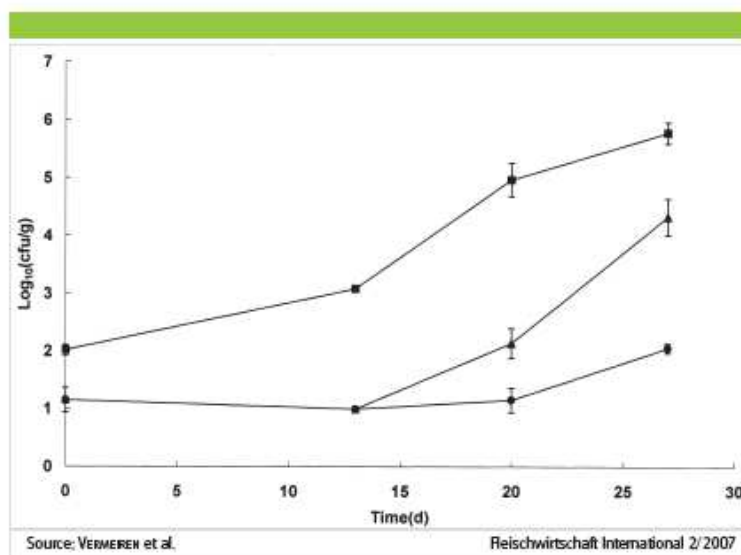


Fig.: The growth of the three-strain *L. monocytogenes* cocktail on vacuum packaged model cooked ham at 7 °C without (■) and with (▲, ●) listeriophage P100 at a dose of 5×10^6 pfu/cm² (▲) or 1×10^7 pfu/cm² (●) (error bars represent standard deviations, n = 2)

vacuum packaged beef, which was stored at 4 °C. The listeriophage had little effect on either of the *L. monocytogenes* strains probably because of the less than optimal bacteria-phage ratio. CARLTON et al. (2005) selected for its work the strictly lytic phage P100 and demonstrated its successful application to control *L. monocytogenes* in artificially contaminated soft cheese.

Materials and Methods

Phage P100 was first isolated eight years ago from a sewage effluent sample taken from a

dairy plant in southern Germany. Samples were tested for the presence of *Listeria* phages. One particular phage formed large clear plaques on most *Listeria* indicator strains and this phage was designated as P100 (CARLTON et al., 2005) and is now proprietary to EBI Food Safety. Most *Listeria* phages seem to be temperate but P100 is one of the few exceptions. The phage belongs to the tailed phages having a contractile tail and an isometric capsid (Myoviridae). This strictly lytic phage has an unusually broad host range within the *Listeria* genus as it is capable to infect and lyse

more than 99% of tested foodborne *Listeria* isolates. EBI Food Safety is the first company that can produce the P100 bacteriophage in a cost-effective and safe way using a strain of the non-pathogenic *Listeria innocua*. The product is sold under the name Listex™ P100.

The effectiveness of Listex-P100 as antilisterial product for RTE meat products was proven at Ghent University through several challenge tests with a cocktail of three *L. monocytogenes* strains (Table 1) on anaerobically packaged CMP.

A preliminary experiment tested the lytic activity of P100 towards *L. monocytogenes* on vacuum packaged cooked chicken fillet stored at 7 °C. The industrially prepared and sliced cooked chicken fillet was treated in three different ways: (1) non-inoculated product (control), (2) product inoculated with a cocktail of three *L. monocytogenes* strains (Table 1) at 10 cfu/g, (3) product inoculated with the same three-strain cocktail of *L. monocytogenes* at 10 cfu/g and subsequently treated with P100 at 1×10^7 pfu/cm². Inoculated products were vacuum packaged and stored at 7 ± 1 °C. At regular time intervals during storage, samples were analysed to determine the number of lactic acid bacteria (LAB), the presence of *L. monocytogenes* (in 25 g) and/or

We operate in the international meat business!

Volume 22
Fleischwirtschaft International
 Journal for meat production and meat processing



Fleischwirtschaft International
 – the English sister of
Fleischwirtschaft

- ▷ News
- ▷ Disseminated on all continents
- ▷ The ideal supplement for transferring competence from Germany to other countries

Fleischwirtschaft International

Fleischwirtschaft International
 Readers' service

D-60264 Frankfurt am Main

Tel ++49 69 7595-1963

Fax ++49 69 7595-1960

E-mail abo-flw@dfv.de

Website www.fleischwirtschaft.com

the number of *L. monocytogenes*.

Further, a more elaborate application test on a sliced model cooked ham (MCH) was performed to confirm the antilisterial effect of P100 and to optimise its dose of application. The model cooked ham (MCH) was an imitation of cooked ham and was produced under very hygienic semi-industrial conditions (in a pilot plant at the Laboratory for Food Chemistry and Meat Technology of Kaho St. Lieven (Gent, Belgium)) resulting in very low contamination level after slicing. This allowed studying the specific interaction between phage P100 and *L. monocytogenes* without having to take into account the background flora. The sliced MCH was either inoculated with the cocktail of the three *L. monocytogenes* strains alone (at 50 cfu/g) or inoculated with the *L. monocytogenes* cocktail (at 50 cfu/g) and subsequently with P100 (at 5×10^6 pfu/cm² or 1×10^7 pfu/cm²). Each treatment was tested in duplicate. At regular time intervals during storage (at 7 ± 1 °C under vacuum packaged conditions), samples were analysed to determine the number of LAB, the presence of *L. monocytogenes* (in 25 g) and/or the number of *L. monocytogenes*. Samples treated with P100 were also subjected to phage number enumeration.

Strains under consideration

Tab. 1: Investigated *Listeria monocytogenes* strains

| Code | LFMFP ¹ -code | Species | Origin/Type |
|------|--------------------------|-------------------------|-------------------------|
| LIS1 | LFMFP 45 | <i>L. monocytogenes</i> | Isolated by LFMFP |
| LIS2 | LFMFP 182 | <i>L. monocytogenes</i> | Scott A (serotype 4b) |
| LIS3 | LFMFP 34 | <i>L. monocytogenes</i> | LMG 13305 (serotype 4b) |

¹ LFMFP – Laboratory of Food Microbiology and Food Preservation, Ghent University
Source: VERBREN et al. Fleischwirtschaft International 2/2007

A final challenge test investigated the performance of P100 in case the treated product is incorrectly vacuum packaged (e.g. incorrect vacuum, packaging film has a too low barrier, leaks, bad seal, etc. allowing oxygen to enter). For this purpose the sliced MCH was either inoculated with the cocktail of the three *L. monocytogenes* strains alone (at 15 cfu/g) or inoculated with the *L. monocytogenes* cocktail (at 15 cfu/g) and subsequently with P100 (at 1×10^7 pfu/cm² or 5×10^7 pfu/cm²). Each treatment was tested in triplicate. After inoculation, products were incorrectly vacuum packaged (vacuum of 20 mbar in stead of 0 mbar) and during subsequent storage at 7 ± 1 °C analysed for the number of *L. monocytogenes*.

Results

In the preliminary experiment on cooked chicken fillet, addition of 1×10^7 pfu/cm² of P100 resulted in a reduction of the *L. monocytogenes* count with

$3.32 \log_{10}$ (cfu/g) compared to the untreated control after 21 days of storage (7 °C, vacuum).

The more elaborate application test on the model cooked ham confirmed the antilisterial effect of P100 (at 7 °C under vacuum) (Fig.). The presence of P100 resulted in a slower growth of *L. monocytogenes*. On the cooked ham inoculated with the cocktail of *L. monocytogenes* and not treated with P100, the number of *L. monocytogenes* increased with $3.77 \log_{10}$ (cfu/g) (mean increase, n=2) over 27 days of vacuum packaged storage, while on cooked ham treated with P100 at a level of 5×10^6 pfu/cm² or 1×10^7 pfu/cm², the number of *L. monocytogenes* increased with 3.18 and 0.91 \log_{10} (cfu/g) (mean increase, n=2), respectively. The difference in antilisterial effect between the two P100 doses was statistically significant (P<0.05). This allowed concluding that treatment with 1×10^7 pfu/cm² of P100 was most efficient to control the growth of *L. monocytogenes* on the MCH.

Immediately after phage treatment (day 0) of the MCH, the *L. monocytogenes* number was significantly (0.5 to 1 \log_{10} cfu/g) lower in the P100-containing samples compared to in the untreated sample and this independent of the applied phage dose (Fig.). This interesting observation could indicate that phage treatment results in an immediate reduction of the number of *L. monocytogenes*. Additional experiments revealed that the initial difference in *Listeria*-number was certainly not due to phage activity on the surface of the ALOA-agar plates during their incubation. A small part of the initial difference might, however, be attributed to the fact that phages immediately after inoculation start to act on the inoculated *Listeria* cells and that in the time during inoculation, sampling and plating (estimated at ± 1 hour) the phage's activity leads to a slightly reduced initial number of *L. monocytogenes* cells.

Results of the final challenge test, investigating the performance of P100 in case the treated product is incorrectly vacuum packaged, are summarised in Table 2. Due to the incorrect vacuum packaging process, oxygen concentrations raised to levels of 10 to 15% after 14 days of storage. This resulted in a rapid growth of *L. monocytogenes* in the control samples since num-

■ Yes, I would like to subscribe to Fleischwirtschaft

Annual subscription rates:

Company _____

Name Mr Mrs _____

Address _____

Postal code _____ Country _____

Tel _____ Fax _____

E-mail _____ Website _____

Date _____ Signature _____

| | |
|------------------|--------------------------------|
| Germany | €80.00 (incl. postage and VAT) |
| EU | €85.60 (incl. postage and VAT) |
| EU (without VAT) | €80.00 (incl. postage) |
| World | €82.00 (incl. postage) |

Fleischwirtschaft International is published 5 times a year. The order is valid immediate effect until cancelled. The period of notice is three months to the end of period.

bers of 2 to 4×10^6 cfu/g were counted after 28 days of storage. On the P100-treated samples, the growth of *L. monocytogenes* was much slower and in particular when treated with the highest dose of 5×10^7 pfu/cm².

Discussion

This study provides evidence on the usefulness of the lytic *Listeria*-specific phage P100 to control *L. monocytogenes* on pre-packaged sliced cooked meat products. Treatment of RTE-meat products with 3×10^7 pfu/cm² of Listex-P100 might contribute to comply with the European food safety objectives for *L. monocytogenes*.

Listex™ P100 was approved as GRAS (Generally Recognized As Safe) by the US FDA on October 17th 2006, based among others on the following scientific and ecological explanations. Bacteriophages are the most numerous life forms on earth, occurring almost everywhere in our environment, in water, in foods of various origin, in the gastro-intestinal tract, etc. (DABROWSKA et al., 2005). On fresh and processed meat and meat products, more than 10^8 viable phages per gram are often present (CARLTON et al., 2005). This means that phages are routinely consumed in quite significant numbers and that mammalian organisms, including humans, are very frequently exposed to interactions with bacteriophages (DABROWSKA et al., 2005). It is commonly believed that bacteriophages cannot infect cells of organisms more complex than bacteria, because of major differences in cell-surface molecules and in key intracellular machinery that is essential for phage replication. CARLTON et al. (2005) studied the P100 genome and conducted an oral toxicity study with P100 in rats and found no indications for any potential risk associated with using P100.

Another risk might be the potential carry-over of undesirable genes by phages between bacterial cells. Indeed, temperate phages are able to transfer virulence properties among bacteria. Their lysogenic status can potentially

Listeriphages effective even with high O₂-levels

Tab. 2: The growth of the three-strain *L. monocytogenes* cocktail on incorrectly vacuum packaged model cooked ham at 7 °C without (control) and with (Exp. 1, Exp. 2) listeriphage P100 at a dose of 1×10^7 pfu/cm² (Exp. 1) or 5×10^7 pfu/cm² (Exp. 2)

| Time (d) | N1 (cfu/g) | N2 (cfu/g) | N3 (cfu/g) |
|---------------------|--------------------|--------------------|--------------------|
| Control | | | |
| 0 | 1.4×10^1 | 7.0×10^0 | 4.5×10^1 |
| 14 | 2.5×10^4 | 2.2×10^4 | 4.0×10^4 |
| 21 | 1.7×10^5 | 3.1×10^5 | 4.6×10^5 |
| 28 | 2.6×10^6 | 2.8×10^6 | 4.2×10^6 |
| Experiment 1 | | | |
| 0 | $<1.0 \times 10^0$ | 3.0×10^0 | $<1.0 \times 10^0$ |
| 14 | 2.0×10^0 | 2.0×10^1 | $<1.0 \times 10^0$ |
| 21 | 7.8×10^2 | 1.1×10^3 | 4.0×10^1 |
| 28 | 6.0×10^2 | - | 1.22×10^4 |
| Experiment 2 | | | |
| 0 | 3.0×10^0 | $<1.0 \times 10^0$ | $<1.0 \times 10^0$ |
| 14 | 1.0×10^0 | $<1.0 \times 10^0$ | $<1.0 \times 10^0$ |
| 21 | 2.0×10^0 | $<1.0 \times 10^0$ | $<1.0 \times 10^0$ |
| 28 | $<1.0 \times 10^0$ | 2.4×10^3 | $<1.0 \times 10^0$ |

Source: VERMEIREN et al.

Fleischwirtschaft International 2/2007

result in the expression of genes encoding properties which increase virulence of the host bacteria. This is however never the case for P100 as it is a strictly lytic (virulent) phage and lytic phages lack the genetic factors required for integration of their phage DNA into the host genome and always enter the lytic cycle and eventually kill and lyse the infected cells. Since P100 is strictly lytic and kills all infected cells, this precludes the opportunity for increased virulence to occur (WHICHARD et al., 2003; CARLTON et al., 2005).

Within the European Union (EU) there is no harmonised legislation that regulates the use of bacteriophages in food products. According to EBI Food Safety, Listex-P100 is a processing aid and not a preservative. EBI claims that the phages in Listex™ P100 are used during the production process of foodstuff for technological purposes, and at a later stage are found in foodstuff only as safe and inevitable residues without having any remaining technological effect on the foodstuff. As a result Listex™ P100 could legally be used as a processing aid for foodstuff under European law. Such processing aids pursuant to European law are exempted from all labelling requirements in connection with the foodstuff they are used for. Only EU member states France and Denmark have specific requirements for the no-

tification of processing aids. Listex™ P100 is the first product on the European market that is containing bacteriophages, meant for application in food products.

The application of Listex P100 does not require special equipment. If the treated product is intended for cooking, as it is the case for cooked meat products, the heat sensitivity of P100 should be taken into account. Because most phages are heat sensitive, they are best applied after the pasteurisation step of cooked meat products in order to guarantee their viability and activity. The meat product can be immersed or dipped into the Listex solution or the Listex solution can be sprayed on the product during or after slicing or in the package immediately before sealing. In the case of spraying an automatic dispensing and spraying device can be used to ensure the correct concentrations of the phage and its even distribution.

References

- Atterbury, R.J., P.L. Connerton, C.E.R. Dodd, C.E.D. Rees and I.F. Connerton (2003): Isolation and characterization of *Campylobacter* bacteriophages from retail poultry, *Applied and Environmental Microbiology* 69, 4511-4518.
- Bell, C. and A. Kyriakides (2005): *Listeria: a practical approach to the organism and its control*, 2nd edition, Blackwell Publishing Ltd, Oxford, 288 p.
- Carlton, R.M., W.H. Noordman, B. Biswas, E.D. de Meester and M.J. Loessner (2005): Bacteriophage P100 for

control of *Listeria monocytogenes* in foods: genome sequence, bioinformatic analyses, oral toxicity study and application, *Regulatory Toxicology and Pharmacology*, 43, 301-312.

4. Dabrowska, K., K. Switala-Jelen, A. Opolski, B. Weber-Dabrowska and A. Gorski (2005): Bacteriophage penetration in vertebrates, *Journal of Applied Microbiology*, 98, 7-13.

5. De Boer, E. (1990): *Listeria monocytogenes* in kip(producten) en vlees(waren). *De Warenchemicus*, 20, 101-108.

6. De Boer, E. and P. van Netten (1990): De aanwezigheid en groei van *Listeria monocytogenes* in pâté, *Voedingsmiddelentechnologie*, 13, 15-17.

7. Dykes, G.A. and S.M. Moorhead (2002): Combined antimicrobial effect of nisin and a listeriphage against *Listeria monocytogenes* in broth but not in buffer or on raw beef, *International Journal of Food Microbiology* 73, 71-81.

8. European Commission (2005): Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs, *Official Journal of the European Union*, L338/1-26.

9. Hudson, J.A., C. Billington, G. Carey-Smith and G. Greening (2005): Bacteriophages as biocontrol agents in food, *Journal of Food Protection*, 68, 426-437.

10. Rijpers, N.P., G. Jannes and L.M.F. Herman (1997): Incidence of *Listeria* spp. and *Listeria monocytogenes* in ready-to-eat chicken and turkey products by polymerase chain reaction and line probe assay hybridisation, *Journal of Food Protection*, 60, 548-550.

11. Thiel, K. (2004): Old dogma, new tricks - 21st Century phage therapy, *Nature Biotechnology*, 22, 31-36.

12. Uyttendaele, M., P. De Troy and J. Debever (1999): Incidence of *Listeria monocytogenes* in different types of meat products on the Belgian retail market, *International Journal of Food Microbiology*, 53, 75-80.

13. Vitas, A.I. and V.A. Garcia-Jalon (2004): Occurrence of *L. monocytogenes* in fresh and processed foods in Navarra (Spain), *International Journal of Food Microbiology*, 90, 349-356.

14. Whichard, J.M., N. Siranganathan and F.W. Pierson (2003): Suppression of *Salmonella* growth by wild-type and large-plaque variants of bacteriophage Felix 01 in liquid culture and on chicken frankfurters, *Journal of Food Protection*, 66, 220-225.

Addresses of authors

Lieve Vermeiren, F. Devlieghere and Johan Debever, Laboratory of Food Microbiology and Food Preservation, Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Gent, Belgium, Lieve.Vermeiren@UGent.be;

Dirk de Meester, Marc Schellekens, Steven Hagens, EBI Food Safety, Johan v. Oldenbarneveltlaan 9, 2582 The Hague, The Netherlands, d.demeester@ebifoodssafety.com